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WOUND HEALING: BIOCHEMICAL PATHWAYS, ULTRASTRUCTURE, AND CLINICAL STUDIES

ANNUAL SUMMARY REPORT

JOHN A. SCHILLING, M.D. AND PATRICK D. GOLDSWORTHY, Ph.D.

July, 1977

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University of Washington
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The insoluble collagens from experimentally induced connective tissue of stainless steel mesh cylinders implanted in man, dog, and rat were analyzed for amino acid composition and essentially no differences were observed, the comparative values for these species being similar. In the rat it was found that these tissues contained three classes of heteropolysaccharides in a complex mixture of glycosaminoglycans, collagen disaccharides, and sialoglycoproteins as well as a less soluble fraction which is more intimately bound to the collagen fibers of tissue. The same three classes of carbohydrate macromolecules were found in the fascia (continued)

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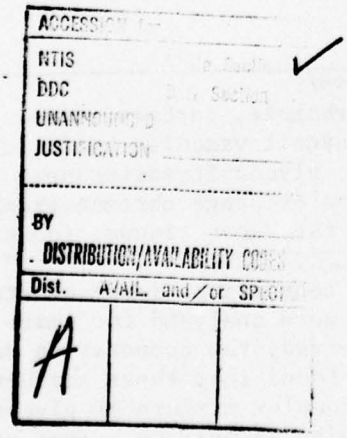
adjacent to the experimentally induced connective tissue. Only hyaluronic acid was found in the adjacent fascia, whereas the connective tissue contained dermatan sulfates and chondroitin sulfates in addition. Dacron Weavenit cylinders were found to provide a unique wound model providing fresh tissue for immediate enzymatic studies.

New methodologies and biochemical procedures were established for the assay of N-acetylgalactosaminyl transferase (AGAT), the biosynthetic enzyme for chondroitin sulfate, which in turn exists in greater proportion than any of the other component glycosaminoglycans of wound tissue. The enzyme assay is based upon its function of transfering UDP-(^{14}D)-N-Acetylgalactosamine to an oligosaccharide acceptor to yield (^{14}C)-N-Acetylgalactosaminyloligosaccharide. The acceptor was prepared by digestion of chondroitin-4-sulfate with hyaluronidase and chromatographic isolation of oligosaccharide.

Stainless steel implanted-cylinder wound models and the healing of skin incision wounds were studied to determine variations in tissue concentrations of AGAT and hydroxyproline during the generation of wound tissue. AGAT concentrations in wound model studies reached a maximum in 2 weeks in wound fluid and were higher than in wound tissue with a maximum at 4 weeks. In wound incision studies AGAT concentrations reached a maximum in 1 week in wound tissue, were higher than in adjacent skin, and corresponded to the maximum concentrations of hydroxyproline, also observed at 1 week in wound and adjacent tissues. The inflamatory effect of turpentine produced increased AGAT concentrations, over controls, in both wound and adjacent tissues.



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REPORT NO. 19

WOUND HEALING: BIOCHEMICAL PATHWAYS, ULTRASTRUCTURE, AND CLINICAL STUDIES

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SUMMARY OF REPORT

The insoluble collagens from experimentally induced connective tissue of stainless steel mesh cylinders implanted in man, dog, and rat were analyzed for amino acid composition and essentially no differences were observed, the comparative values for these species being similar. In the rat it was found that these tissues contained three classes of heteropolysaccharides in a complex mixture of glycosaminoglycans, collagen disaccharides, and sialoglycoproteins as well as a less soluble fraction which is more intimately bound to the collagen fibers of tissue. The same three classes of carbohydrate macromolecules were found in the fascia adjacent to the experimentally induced connective tissue. Only hyaluronic acid was found in the adjacent fascia, whereas the connective tissue contained dermatan sulfates and chondroitin sulfates in addition. Dacron Weavenit cylinders were found to provide a unique wound model providing fresh tissue for immediate enzymatic studies.

New methodologies and biochemical procedures were established for the assay of N-acetylgalactosaminyl transferase (AGAT), the biosynthetic enzyme for chondroitin sulfate, which in turn exists in greater proportion than any of the other component glycosaminoglycans of wound tissue. The enzyme assay is based upon its function of transfering UDP-(^{14}C)-N-Acetylgalactosamine to an oligosaccharide acceptor to yield (^{14}C)-N-Acetylgalactosaminyloligosaccharide. The acceptor was prepared by digestion of chondroitin-4-sulfate with hyaluronidase and chromatographic isolation of oligosaccharide.

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FOREWORD

There have been all too few definitive studies following the progress of the wound and at the same time correlating changes in biochemical constituents. This has been due to lack of suitable wound models. Polyvinyl sponge implants have been used extensively but leave much to be desired in the way of normal type wound tissue. The stainless steel mesh cylinder implant, in the opinion of this laboratory, more closely simulates the uncomplicated wound tissue structure. A great deal of work has been carried out in this laboratory with experimental animals and the stainless steel cylinder techniques. Both the detailed fine structure using E.M. techniques and the biochemical alterations in tissue content during healing have been assayed extensively. The correlation of biochemical content with structure, function and metabolism of the wound is imperative. The use of more specific radioactive precursors of wound metabolites can and must be explored in this area. Studies of this wound model in human subjects have already been carried out successfully in this laboratory. Application of the enzymatic assay, as a postulated monitor of actual wound healing in human, may ultimately be made. In anticipation of this, the wound model studies in dogs need to be repeated on skin incisions in dogs.

We have used a simple, reproducible experimental wound model to accumulate a vast amount of information concerning wound fluid, fibroplasia, and collagen-matrix formation in rat, dog, and man. The wound is induced by a stainless steel wire mesh cylinder implanted subcutaneously into the host with sterile techniques. The implanted cylinder itself evokes very little foreign-body inflammatory response. The size of the cylinder, mesh of the wire, and its gauge may be varied appropriately. In essence, the implanted cylinder creates a sterile dead space which fills with an extracellular plasma-like fluid and with fibrocollagenous tissue in an orderly and reproducible fashion. The wound-induced connective tissue and component fluid may be sampled from the cylinder at any time during the time-course of fibroplasia. Tracer materials such as ^{14}C or ^{3}H labeled hexosamines, hexoses, or amino acids may be injected directly into the cylinder and followed out into the whole animal body. Conversely, radioactively tagged precursors may be injected systemically and followed within the cylinder where they are incorporated or bound. The "wound fluid" inside the cylinder as well as the fibrocollagenous tissue therein may be removed and analyzed qualitatively and quantitatively for chemical content, and specific components may be isolated for radioactivity measurements. The connective tissue may also be removed fresh and intact from inside the cylinder wall and assayed by explanting into tissue culture, or it may be studied for its enzyme content or by electron microscopy and electron microscope autoradiography. The wound fluid and fibrocollagenous tissue inside the cylinder may be compared with the body fluids, boood serum, or body tissues outside the cylinder. The model is not complicated by infection, contracture, or epithelialization. It is analogous to a tissue culture <u>in vivo</u> of the fibroblast, a highly anabolic unit, which produces collagen and some of the glycosaminoglycans of the ground substance.

Collagen the binding tissue of all mammals is a key material in wound healing, without which there is no strength permenance. It is not inert but shares in the dynamic balance of local and whole body stress, nutrition, and metabolism. Collagen contains a small percentage of disaccharide side-chains thought to serve both as intermolecular bridges and play a significant role in the maturing of the collagen fibres. The collagen fibres are imbedded in an amorphous carbohydrate matrix. This matrix has been assigned no definite structure by present electron microscope techniques, but is known to have a complex chemical nature consisting of acidic glycosaminoglycans (mucopolysaccharides) and glycoproteins (proteins with covalently bound heteropolysaccharide chains). The matrix portion of the collagen unit is obviously important in the synthesis, maturation, and metabolism of connective tissues and the healing of wounds.

The specific aims of these studies are to contribute new knowledge, to utilize established techniques, to assay appropriate new information and to apply whenever possible this knowledge to the care and treatment of wounds. Specifically, this will involve:

1. Continuation of the biochemical studies in the rat and dog utilizing research methods developed over a number of years whereby wound connective tissue can be procured with the stainless steel wire mesh model and examined throughout its time-course of development. Also the tissue in the outer capsule of the wound model as well as tissue adjacent to the would will be examined by the same biochemical assays and these data will be compared to that of wound tissue itself, Similar comparisons of tissue, both proximal and distal to the healing wound tissue of skin incisions will be made in dogs. The influence of topical agents, locally applied to the incision, can be compared with untreated incisions in the same animal. The influence and importance of the cells and tissue neighboring the wound has been neglected in this study and must now be investigated since wound connective tissue may be the end-product of these cells.

2. Examination will be made of wound tissue and surrounding tissues in the wound locale for certain enzymes which may be involved in the initial inflammatory response and in the healing phase of the wound. Initially, measurements will be made of the activity of galactosaminyl transferase which has been observed to be proportional to the presence of chondroitin sulfate -- the major glycosaminoglycan component of wound connective tissue.

Each of the following sections in this report is complete in itself, containing a brief background, pertinent methods, results and discussion, summary, and bibliography.

TABLE OF CONTENTS

BIOCHEMICAL INVESTIGATION OF THE HEALING WOUND

A. Previous Studies (1953 - 1973) in the Biochemical Section

Wound Model: The continued success of our wound healing studies has been due mainly to the use of a unique, yet simple, and reproducible experimental wound model (1,6,15). A considerable amount of basic information has been obtained about the response in animals to implanted stainless steel mesh cylinders. We have also studied the implantation of these cylinders in man. This remarkably inocuous wound study technique has provided sufficient amounts of wound connective tissue for biochemical analyses for extended periods of regeneration of the wound. These studies have probably constituted the first efforts to structure and study wound tissue in the human.

Wound fluid: It was noted in early studies (1-3,5,9) that a fluid quickly filled the empty space within the implanted cylinders. This fluid was examined and found to be similar in many respects to the serum of the animal. It differed from serum in its increased quantity of albumin and glycoprotein and decreased content of globulins of high molecular weight. It thus appeared to be a serum exudate of specific nature. These results focused attention upon the possible implication of glycoproteins in the process of wound healing (8).

Wound tissue: Considerable amounts of a fibrous tissue were noted to accumulate within cylinders implanted for long periods of time. Studies were instigated in dogs where large quantities of fluid and tissue could be obtained with multiple cylinder implantations (5). As a corollary to glycoprotein changes noted in wound fluid, variable changes were observed in glycosaminoglycans of wound tissue (8). Methods were developed in our laboratory (11) to isolate these carbohydrate substances and separate them by ion-exchange chromatography. Direct quantitation and analysis has been carried out in tissue varying in developmental age from 2 weeks to 8 months. The dominant glycosaminoglycan of wound tissue is chondroitin sulfate. Small amounts of hyaluronic acid are also found. While these types appear to remain qualitatively the same, their absolute amounts decrease with the age of the tissue. The mucopolysaccharides constitute only one-fourth of the total structural carbohydrates. Part of the other three-fourths carbohydrate is composed of sialic acid containing glycoproteins. It is now thought that these glycoproteins may play a more dynamic role in the development of the tissue (16). A definite interrelationship between collagen and structural carbohydrates is emerging from the studies of the biochemical composition of wound tissue.

Metabolic studies: The wound fluid-wound tissue cylinder unit is an excellent system for metabolic studies with radioactive substances. These have provided valuable information concerning the origin and use of specific metabolites needed during the healing of the wound. To study the biosynthesis and metabolism of carbohydrate substances during healing, our laboratory was one of the first to utilize 1- ^{14}C-glucosamine (10). When given intra-

peritoneally, glucosamine is incorporated first in the liver for synthesis of serum glycoproteins. These substances eventually reach the wound site and are utlized by cells undergoing synthesis of wound tissue. The incorporation of glucosamine occurs also in acid mucopolysaccharides of wound tissue of the rat (11,13). The injection of radioactive glucosamine directly into the wound capsule stimulates its incorporation tenfold. Incorporation of glucosamine in vitro by wound connective tissue and the incorporation of glucosamine by connective tissue in hepatectomized rats has been demonstrated (11). This evidence shows that carbohydrate macromolecules are dynamically involved in wound healing and synthesis of these substances does not take place locally at the wound site and is not dependent upon metabolites from the liver and elsewhere.

We reported the first use of 1-^{14}C-galactosamine (15). Its utilization in the rat parallels that of glucosamine in the liver but is incorporated at a reduced rate by wound tissue. The metabolic pathways and enzyme kinetics involved in galactosamine metabolism apparently differs in liver and wound tissue. Glucosamine can be converted to galactosamine at the acetylated dinucleotide (UDP-N-acetylglucosamine) level, but galactosamine must first be converted to glucosamine in order to be utilized in this pathway. The metabolism of galactosamine in the wound is still unclear at this time and requires further study.

A metabolic study using sulfur-35 and a study with radioactive glucosamine, glycine, and proline in unison was carried out in dogs (12, 17). The experiments, designed to study the synchronous metabolism of collagen and ground substance of wound tissue, showed that these intraperitoneally administered radioisotopes were transported to the wound site via the serum and wound fluid. This study illustrated the dominant role of serum alpha-1 globulins in wound healing.

Studies in the human: Knowledge gained over the years from studies with research animals was applied to the study of this wound model in man. Wound tissue harvested from cylinders of human subjects was analyzed for lipid, water, dry weight, total protein, collagen, hexosamines, hexoses, hexuronic acids, and sialic acids. The results were compared in relation to the age of the tissue during its synthesis in vivo and with previous and concurrent investigations in the dog and rat (18-21). Analytically, there was a general similarity in this scar-type wound connective tissue in all species studied. Of note was the depolymerization of collagen in tissue of cylinders implanted in man for periods of 12 weeks or longer. This was not noted in dogs or rats. The carbohydrate content of this tissue was quantitatively similar in all three species. Although carbohydrate material makes up only 3 percent of the dry weight of the tissue, time-related changes in various carbohydrate constituents were noted to be of paramount importance to the regeneration and integrity of this tissue. Many proteins are now known to contain small amounts of carbohydrates, but their function is not well understood in many cases. However, the cell envelopes of mammalian cells have been found to contain sialoglycoproteins in the outmost region, and one of their functions is thought to be that of cell adhesion. Sialic acid, the sialoglycoprotein index of wound connective tissue, remained at a constant level,

but the significance of this is unknown. The importance of these sialoglycoproteins is evidenced by their presence 3 times in excess of the acid mucopolysaccharides (glycosaminoglycans). Over one-half of the carbohydrate of wound tissue was in the nature of neutral sugars found not only in glycoproteins but also in the structural protein collagen. Neutral sugars (pentoses) have been found in the protein-carbohydrate linkage of several mucopolysaccharides. Of special interest in this regard was the high content of glucose in human wound connective tissue. Part of this glucose is involved in the glucose-galactose disaccharide sidechains of collagen, but that found in excess of galactose may originate in glycoproteins of unknown composition.

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E. Recent Studies (1973-1974) of Insoluble Collagen of Experimentally Induced Connective Tissue of Man, Dog, and Rat.

Although much is now known about the chemistry of collagen most, studies so far have dealt with soluble collagen, which accounts for only a small portion of the total collagen in most tissues. It was decided to prepare and analyze insoluble collagens from experimentally induced connective tissue of man, dog, and rat and to determine the differences in the amino acid composition in these tissues.

The insoluble collagens from experimentally induced connective tissue of man, dog, and rat were analyzed for amino acid composition (1,2). The insoluble collagens were prepared by first extracting the tissue with water and sodium acetate to remove carbohydrate substances. Soluble collagen was extracted with sodium citrate buffer and the resulting insoluble collagen remained. Essentially no differences were seen in the amino acid content of the insoluble collagens of the experimentally induced connective tissue of man, dog, or rat. A higher number of hydroxylysine residues were recorded for this wound tissue than that found in the dermis, but the comparative values for man, dog, and rat were similar. Wound connective tissue was found to be unique in its hydroxylysine quantity and is indicative of a higher quantity of hydroxylysine-linked collagen disaccharides for this tissue as compared to the dermis. The insoluble collagens of the wound tissues may still have some non-collagenous materials present since the glycine, proline, and hydroxyproline are lower than expected for collagens. Also the leucine, phenylalanine, and tyrosine are a little higher than expected. On the other hand, these values may be characteristic for wound tissue as similar findings were previously reported by other workers with guinea pig scar collagens.

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C. Recent Studies (1973-1974) of the Glycopeptides of the Matrix of Experimentally Induced Connective Tissue of the Rat

Synthesis of connective tissue within stainless steel wire mesh cylinders has been induced by their subcutaneous implantation in man, dog, and rat. This specific type of connective tissue has been found to contain, in addition to collagen, about 3 percent carbohydrate in the form of hexuronic acid, glucose, galactose, mannose, glucosamine, galactosamine, fucose, and sialic acid (1). Further investigation of the structural units of these carbohydrates (2) has revealed the diverse nature and complexity of the heteropolysaccharides of this tissue.

Investigation of the structural carbohydrate units of experimentally induced connective tissue was carried out in the rat. Three classes of heteropolysaccharides found in this tissue are glycosaminoglycans, structural sialoglycoproteins, and collagen disaccharide side-chains. Studies were conducted to do a mild extraction of the connective tissue to remove the more soluble glycopeptides and then subject the remaining tissue to enzyme digestion and critical examination of the remaining less soluble glycopeptides by filtration gel separation. The digestibility of trypsin and pronase both together and by pronase alone was analyzed. It was found that a considerable amount of glycoprotein material could be removed from the tissue by water extraction alone. Most of the water soluble components were removed in the first 24-hour extraction by this method. Electrophoretic studies of the water extract revealed that serum-type glycoproteins were present in this fraction. Sephadex G-50 separation of the water extract of the tissue after pronase digestion showed this water extract to contain only sialoglycopeptides. The amount of these sialoglycopeptides removed by water extraction was estimated to be about two-thirds of the total sialoglycopeptide of the tissue. The wound tissue does still contain more difficulty soluble sialoglycopeptides which amount to only one-third that previously determined. It was concluded that the experimentally induced connective tissue of stainless implanted cylinders contains three classes of heteropolysaccharides. These are a complex mixture of glycosaminoglycans, collagen disaccharide side-chains, and sialoglycoproteins. Some of the latter originate from the blood of the animal, or extracellular fluid, and these can easily be extracted by water. There still remains a less soluble fraction which is more intimately bound in the collagen fibres of the tissue. These less soluble sialoglycoproteins have been reported in other types of connective tissue. The function of any or all of the heteropolysaccharides in connective tissue is still speculative at this point.

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D. Recent Comparative Studies (1973-1974) of Wound Connective Tissue and Adjacent Host Connective Tissue in the Dog and the Rat.

Stainless steel wire mesh cylinders were implanted in the subcutaneous region of the back of rats and a unique wound-type connective tissue induced on the interior and exterior surfaces of the implanted wire mesh cylinders (1,2). The cells which populate the subcutaneous regions of the back and which synthesize the connective tissue are the differentiated forms of the fibroblast. We have attempted to study the connective tissue from the cylinders and compare it to tissue in the adjacent fascia in the same region in a biochemical assay. We have separated the carbohydrate portion of these connective tissues by Sephadex filtration following enzyme digestion to free the material of collagen. The same three classes of carbohydrate macromolecules were found in the connective tissue of the cylinder and in the adjacent fascia. The main differences appear to be in the glycosaminoglycan carbohydrate class. Only hyaluronic acid was found in the adjacent fascia, whereas connective tissue of the cylinder contained in addition dermatan sulfates and chondroitin sulfates. The reason for the difference in glycosaminoglycan content appears to be one of function. The adjacent fascia serves in a somewhat lubricant fashion between the dermis and musculature. The wound connective tissue serves a more protective function whereby the cylinder is walled off or encapsulated from the host.

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E. Recent Studies (1973-1974 of the Feasibility of Other Wound Models: The Dacron "Weavenit" Vascular Prosthesis

The production of granulation tissue for studies of wound healing has been accomplished by a variety of models over the years (1-7). We have used the stainless steel wire mesh cylinder in this regard for many fruitful studies of wound tissue (8). However, in search for a prosthesis which stimulates production of connective tissue for use in the fresh state, we have tested a Dacron vascular graft material (9) in this year's work. The weaved Dacron cylinder was implanted in a series of rats and was found to produce an avascular connective tissue tube within the prosthesis which could be removed in tube shape for immediate studies of fresh tissue. The

amount of tissue within the Dacron cylinder was found to increase in amount with the time period of implantation, and at 7 weeks a sample of fresh tissue from a single cylinder weighed 200 mg. It was concluded that the Dacron Weavnit cylinder provides a unique wound model which should be investigated further as an aid to the study of wound connective tissue in fresh form probably for enzyme studies.

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F. Current Studies (1975-1977) of Enzymes Involved in Wound Healing

INTRODUCTION

Our understanding of the intracellular location of carbohydrate attachment to the peptide portion of glycoproteins has aided studies concerning the structural makeup of these substances. The enzymes responsible for synthesis of the carbohydrate portion are located on the membranes of the endoplasmic reticulum of the cell and the assembly of the carbohydrate units take place by a series of glycosyltransferases functioning to transfer activated sugars from the nucleotide derivatives to appropriate acceptors (1). The synthesis of the carbohydrate portion of proteoglycans is not subject to direct genetic control since the carbohydrate units are added postribosomal but rapid physiological control can be exerted on these substances by control of enzyme activity. The absence of an enzyme of this type could result in a pathological condition, i.e., connective tissue disease and/or poor wound healing response in the host. It is obvious that the investigation of glycosyltransferases would provide an important new dimension for the studies of wound healing. The timing of synthesis or activation of these enzymes would be crucial for the proper development and organization of the involved tissues. Chondroitin sulfates exist in greater proportion than any of the other component glycosaminoglycans of wound tissue, therefore the study of the chondroitin sulfate biosynthetic enzyme, N-acetylgalactosaminyltransferase (AGAT), would yield an index of biosynthesis of these compounds.

METHODS

Even-numbered oligosaccharides with nonreducing terminal glucuronic acid were prepared as substrate acceptors for N-acetylgalactosaminyltransferase. The polysaccharide was digested with hyaluronidase and the oligosaccharide products separated by ion exchange and Sephandex gel chromatography (2). Whale cartilage chondroitin-4-sulfate (Miles Laboratories, Inc., Kankakee, Illinois) in sodium acetate buffer, (pH 5.0, containing 0.15 M NaCl) was incubated at 37°C for 24 hours with testicular hyaluronidase (Aktiebolaget Leo, Helsingborg, Sweden). The hyluronidase digest solution was clarified by centrifugation and aliquots of oligosaccharide were successively applied to a column of ion exchange resin (Dowex 1-X8, 200-400 mesh, Cl^-cycle). A non-linear elution gradient of lithium chloride was pumped into the top of the column.

The hexuronic acid concentration in each fraction was determined by a modified carbazole procedure (3). The fractions thus identified as constituting each eluted peak were pooled, lyophylized desalted on a column of Sephadex G-25 relyophilized for use as oligosaccharide acceptors in AGAT assay.

The wound-model studies are carried out in 20-35 kg dogs by implanting with a trocar 12 stainless steel mesh cylinders (10 mm diameter X 70 mm length, closed at both ends) in each dog into 12 separated subcutaneous pocket-areas, radiating laterally from two dorsal midline incisions.

The cylinders, along with portions of the fascia in the subcutaneous area surrounding the cylinder, are removed singly or in pairs at intervals varying up to 30 or 100 days following cylinder implantation. Four tissues are isolated from each cylinder: Wound fluid, encapsulated within the cavity of the cylinder, is removed immediately by syringe and needle; Outer wound tissue is peeled off the outer surface of the cylinder; Inner wound tissue, encapsulating the wound fluid is scraped from the inner surface of the cylinder after the cylinder is cut open; Mesh wound tissue is recovered by lyophilizing the mesh-embedded tissue and removing the mesh wire by wire, from the dried tissue. Three equal wet tissue aliquots are taken: One which is homogenized in HEPES buffer and assayed for the enzyme N-acetylgalactosamino transferase (AGAT) by the Method of Tesler, Robinson and Dorfman (4); A second which is oven-dried to remove water, and petroleum ether-extracted, to remove fat, and then assayed for hydroxyproline (5); And the third which will be extracted with water to remove the glycoproteins which are probably not of connective tissue origin which can be studied separately. Following this extraction, the tissue will be digested with Pronase which alone adequately releases essentially all glycopeptide material. The enzyme digest will then be filtered to remove the insoluble collagen portion and the filtrate will be subjected to Sephadex G-50 separation using 0.5 M pyridine-acetate of pH 5.0 for elution. The column fractions will be monitored for amino acid content by a ninhydrin method (6), for hexose by an anthrone method (7), for sialic acid by a thiobarbituric acid method (8), and for uronic acid by a carbazole method (3).

The skin incision wound studies are carried out on 20-35 Kg dogs by making twelve 25 mm longitudinal, subcutaneous, dorsal incisions, six on each side, 76 mm from the midline, 76 mm between centers, and extending 460 mm overall from the vicinity of the scapular spine to the iliac crest. Biopsies of each entire incision site, including wound healing tissue, along with some adjacent skin and subcutaneous area are removed singly or in pairs at intervals varying up to 30 or 100 days following the incision. Three equal wet tissue aliquots are assayed for AGAT enzyme, hydroxyproline, and glycopeptides as described for the wound model experiments.

In rats the wound model studies are conducted as in the dogs except only 2 cylinders per rat are inserted by Russian forceps. The wound incision studies also are similar to those in dogs except only one incision is made on each side.

We have recently employed dacron "Weavenit" vascular prostheses in rats in the same manner as with metal type cylinders. The tissue in the dacron cylinders can be removed in tube form in the fresh state at the time of excision. We feel this fresh tissue can possibly be used directly for enzyme studies following homogenization and differential ultracentrifugation.

The enzyme N-acetylgalactosaminyl transferase (AGAT) studies are carried out on fresh wet wound tissue from the cylinders on the incision sites as well as on the tissues surrounding the cylinders or wounds. The enzyme AGAT is extracted from the tissue which is homogenized in a Sorvall Omnimixer (10 ml minimum volume) or Tekmar Tissumizer (1-2 ml minimum volume) in a buffered (pH6.5) cold solution (2-N-morpholinoethane sulfonic acid containing KCl, Mn Cl_2 and $MgCl_2$). The cell particulate fractions are separated by differential ultracentrifugation at 10,000g and 105,000g. The AGAT assays are made on both the 105,000 pellet and supernatant using the radioactive nucleotide donor (UDP-N-14_C-galactosamine) which is transferred by the enzyme to the added oligosaccharide substrate acceptors and is carried

out by the method of Roden et al (4). The substrate is chondroitin 4- or 6-sulfate (Miles Laboratories) or the hexasaccharide isolated from these after digestion with testicular hyaluronidase. The enzyme levels observed during the time-course of wound healing will be correlated with those of hydroxproline, glycopeptides, and glycosaminoglycans.

RESULTS AND DISCUSSIONS

Studies of wound healing depend almost entirely upon the adequacy of the wound model. Biochemical studies of developing connective tissue of the wound in this laboratory will continue using the stainless steel wire-mesh cylinder wound model. This model simulates the uncomplicated wound because when implanted subcutaneously it induces the synthesis of scar-type connective tissue. The model invokes the synthesis of tissue in man which appears chemica-ly the same as that in dog or rat, (9,10) and thus may ultimately aid in the diagnosis and treatment of human connective tissue diseases. Another feature of this implant model is that it provides what is considered to be a new and normal-type connective tissue which is free from the old adjacent host tissue.

While comparative studies of the wound model and skin-incision wounds will be continued, primarily in the dog, some additional studies will be conducted in the rat as well. Wound tissue, obtainable at four weeks post-implantation from 12 cylinders in a single dog, can yield as much as 1.3 gm (dry weight) compared to a yield of 1.7 gm (dry-weight) from 12 cylinders implanted in 6 rats. However, larger amounts of wound tissue are observed in the cylinders from dogs at early time periods than in those from rats. The biopsy of 12 entire incision sites plus adjacent tissues yield 30 grams of wet tissue from a single 30 kg dog. Thus, to minimize the biologic variables inherent in studying a group of rats, observations will be made of multiple tissue samples, taken sequentially or at one time, from a single dog or made of locally applied influences on wounds relative to untreated control wounds in the same animal. In vivo radioisotope studies should yield isolated labeled glycopeptides and glycosaminoglycans and their metabolic precursors of higher specific activity in rats that in dogs because of less dilution in the smaller animal. Thus, the economic tradeoff, against biologic variation, of using rats, rather than dogs, will have to be explored by administering ^{14}C-glucosamine and ^{14}C-galactosamine in pilot experiments with dogs.

Our understanding of the intracellular location of carbohydrate attachment to the peptide portion of glycoproteins has aided studies concerning the structural makeup of these substances. The enzymes responsible for synthesis of the carbohydrate portion are located on the membranes of the endoplasmic reticulum of the cell and the assembly of the carbohydrate units take place by a series of glycosyltransferases functioning to transfer activated sugars from the nucleotide derivatives to appropriate acceptors (1). The synthesis of the carbohydrate portion of proteoglycans is not subject to direct genetic control since the carbohydrate units are added postribosomal but rapid physiological control can be exerted on these substances by control of enzyme activity. The absence of an enzyme of this type could result in a pathological condition, i.e., connective tissue disease and/or poor wound healing response in the host. It is obvious that the investigation of glycosyltransferases would provide an important new dimension for the studies of wound healing.

The timing of synthesis or activation of these enzymes would be crucial for the proper development and organization of the involved tissues. Chondroitin sulfates exist in greater proportion than any of the other component glycosaminoglycans of wound tissue, therefore the study of the chondroitin sulfate biosynthetic enzyme, N-acetylgalactosaminyl transferase, would yield an index of biosynthesis of these compounds.

The initial three months period of the resumption of the biochemical investigations of the Wound Healing Studies Project, subsequent to the arrival on the Project of a biochemist Research Associate Professor Patrick D. Goldsworthy, was required to carry out the planning, organization, extablishment, and reactivation of this Project in a new laboratory. Research in the newly activated laboratory first established new methodologies and biochemical procedures for assaying N-acetylgalactosaminyltransferase according to the following scheme (2):

$$\left.\begin{array}{l}\text{UDP-}(^{14}\text{C})\text{-N-Acetylgalactosamine} \\ \text{oligosaccharide (acceptor)} \\ \text{N-acetylgalactosaminyl transferase}\end{array}\right\} = (^{14}\text{C})\text{-N-Acetylgalactosaminyloligosaccharide}$$

During the period of this report 12 dogs varying in weight from 19-35 kg, were used in stainless steel implanted cylinder wound model and skin incision wound healing studies of N-acetylgalactosaminyl transferase and hydroxyproline concentrations in wound and adjacent tissues.

The concentrations of N-acetylgalactosaminyl transferase (AGAT), measured in the wound model studies, were observed (Fig. 1) to be: highest in wound fluid, reaching a maximum by the 14th day and declining to baseline by the 42nd day; Intermediate in the inner wound tissue, having reached a maximum by the 27th day; and the lowest in outer wound tissue, declining from a maximum at 14 days to base line by 28 days.

Measurements of AGAT, in the wound incision studies, were found (Fig. 2) to be higher in the wound tissue than in the adjacent tissue and reaching a maximum by the 8th day and declining to base line by the 22nd day.

The hydroxyproline concentrations, in the wound incision studies, were slightly lower in the wound tissue than the adjacent tissue and both reached maximum values at 8 days (Fig 3.) corresponding to maximum AGAT concentration in wound tissue (Fig. 4) and adjacent tissue (Fig. 5).

The inflamatory effect of turpentine, in wound incision studies was observed to produce equal AGAT concentrations in both wound and adjacent tissues (Fig. 6), while increasing AGAT concentrations, over controls, in both wound tissue (Fig. 7) and adjacent tissue (Fig. 8).

Additional parameters of comparison with AGAT will shortly become available as we proceed to collaborate with studies by Dr. John E. Olerud, (Department of Medicine, University of Washington) to assess wound healing by comparing standard invasive techniques, including tensil strength testing, hydroxyproline determinations, and structural morphology, with a proposed new noninvasive technology utilizing ultra sound and controlled application of mechanical force.

FIGURE 1

N-ACETYLGALACTOSAMINYL TRANSFERASE

WOUND MODEL STUDY

DOG 1

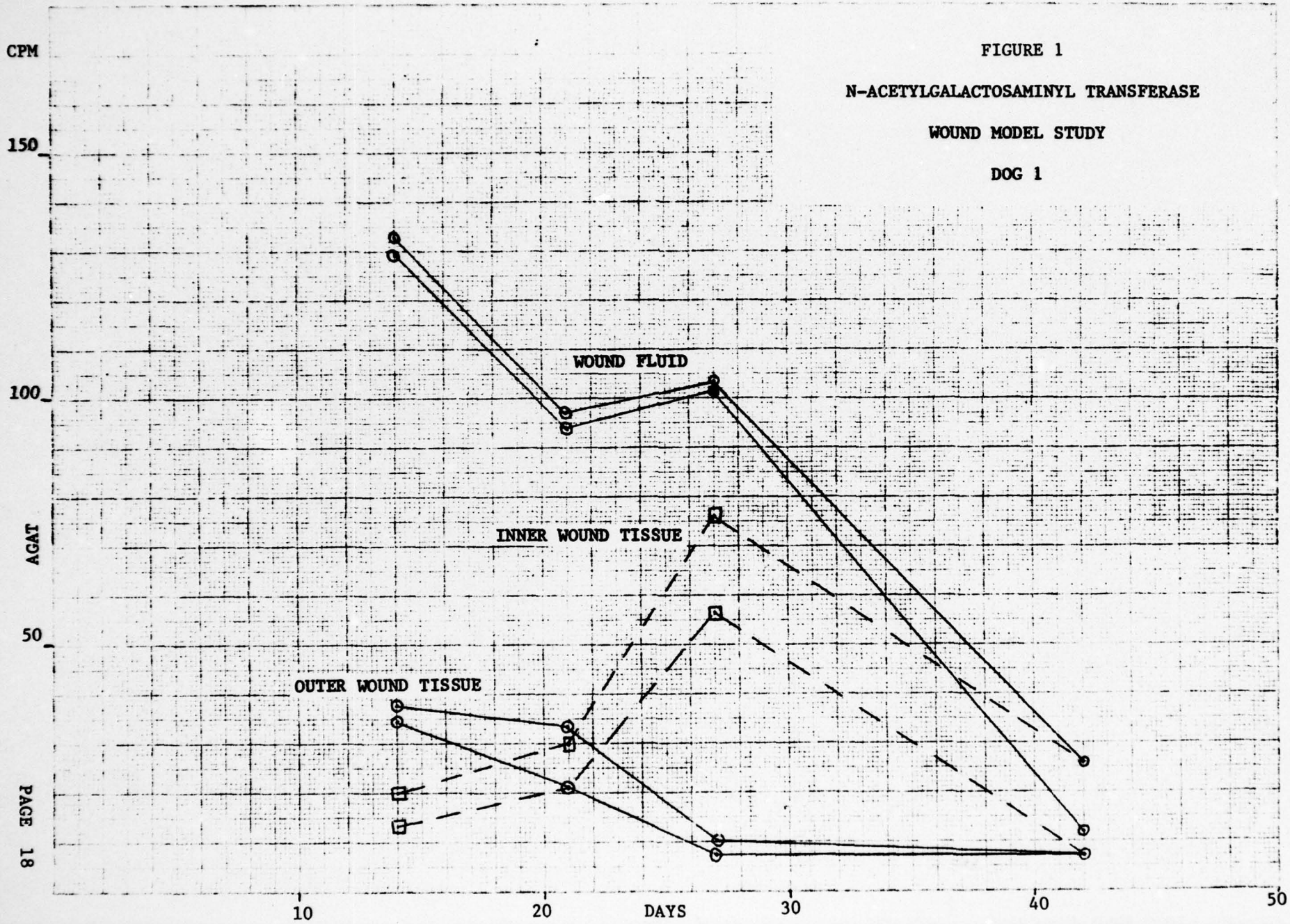
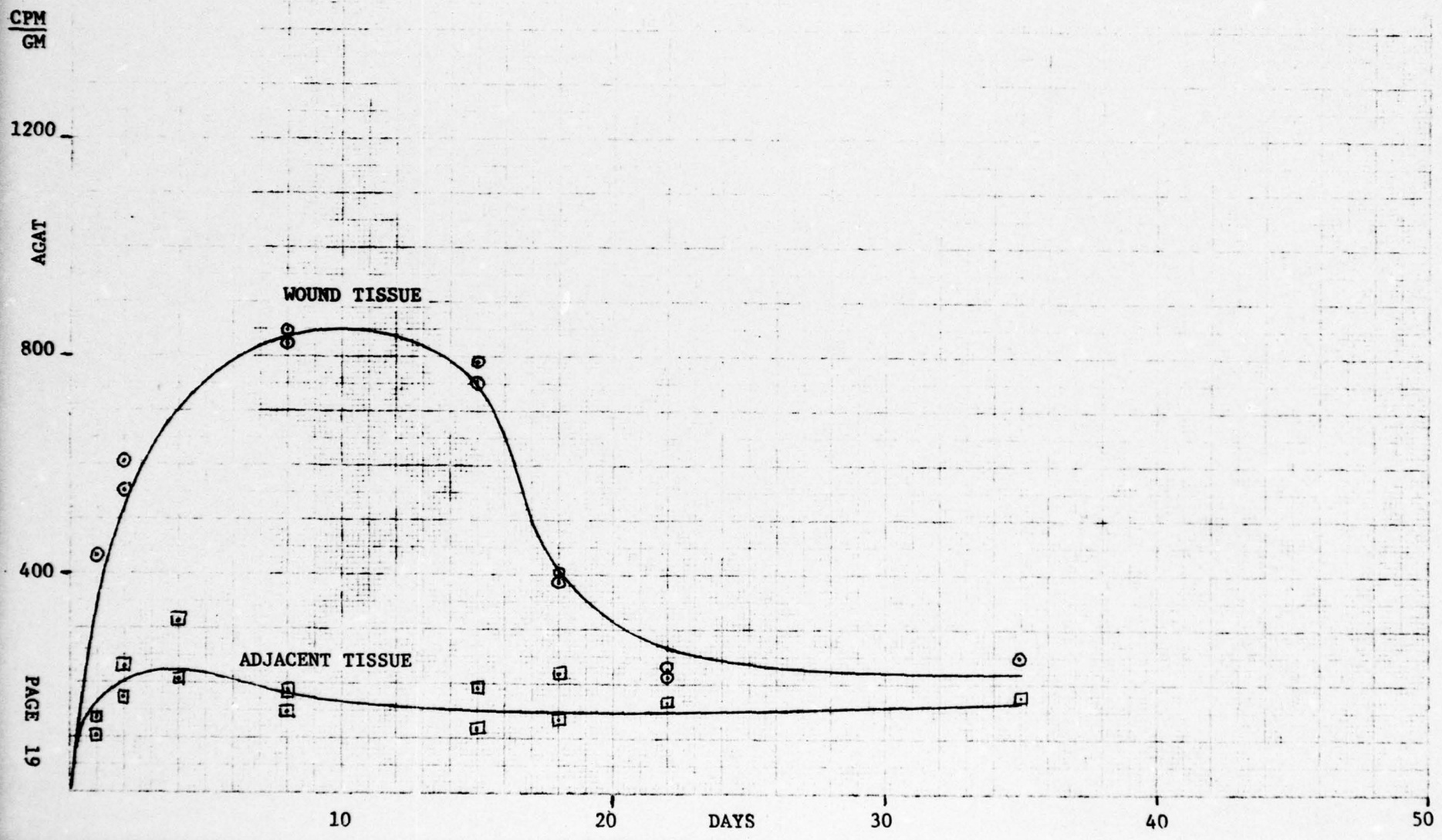


FIGURE 2

N-ACETYLGALACTOSAMINYL TRANSFERASE

WOUND INCISION STUDY

DOG 8



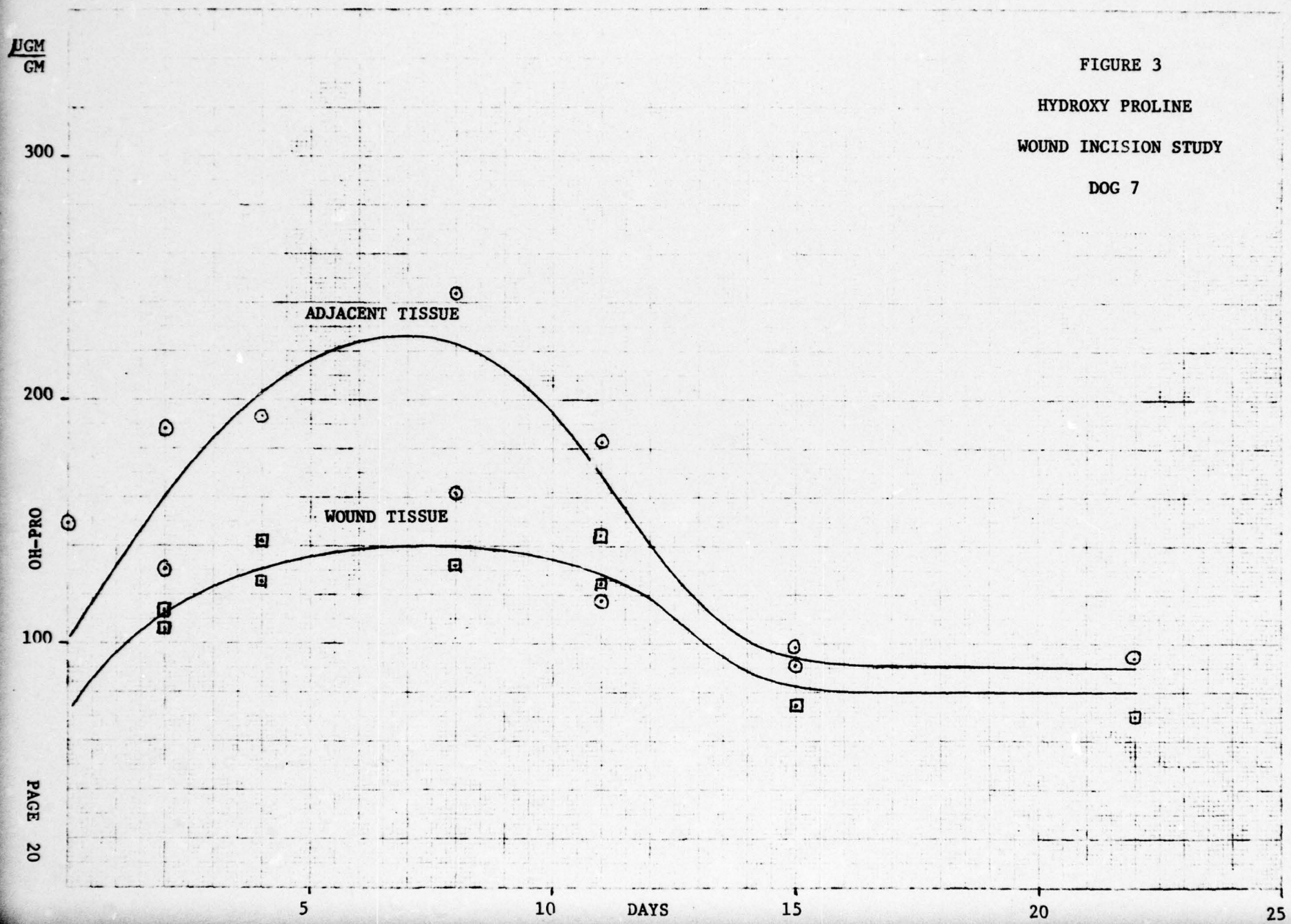


FIGURE 3

HYDROXY PROLINE

WOUND INCISION STUDY

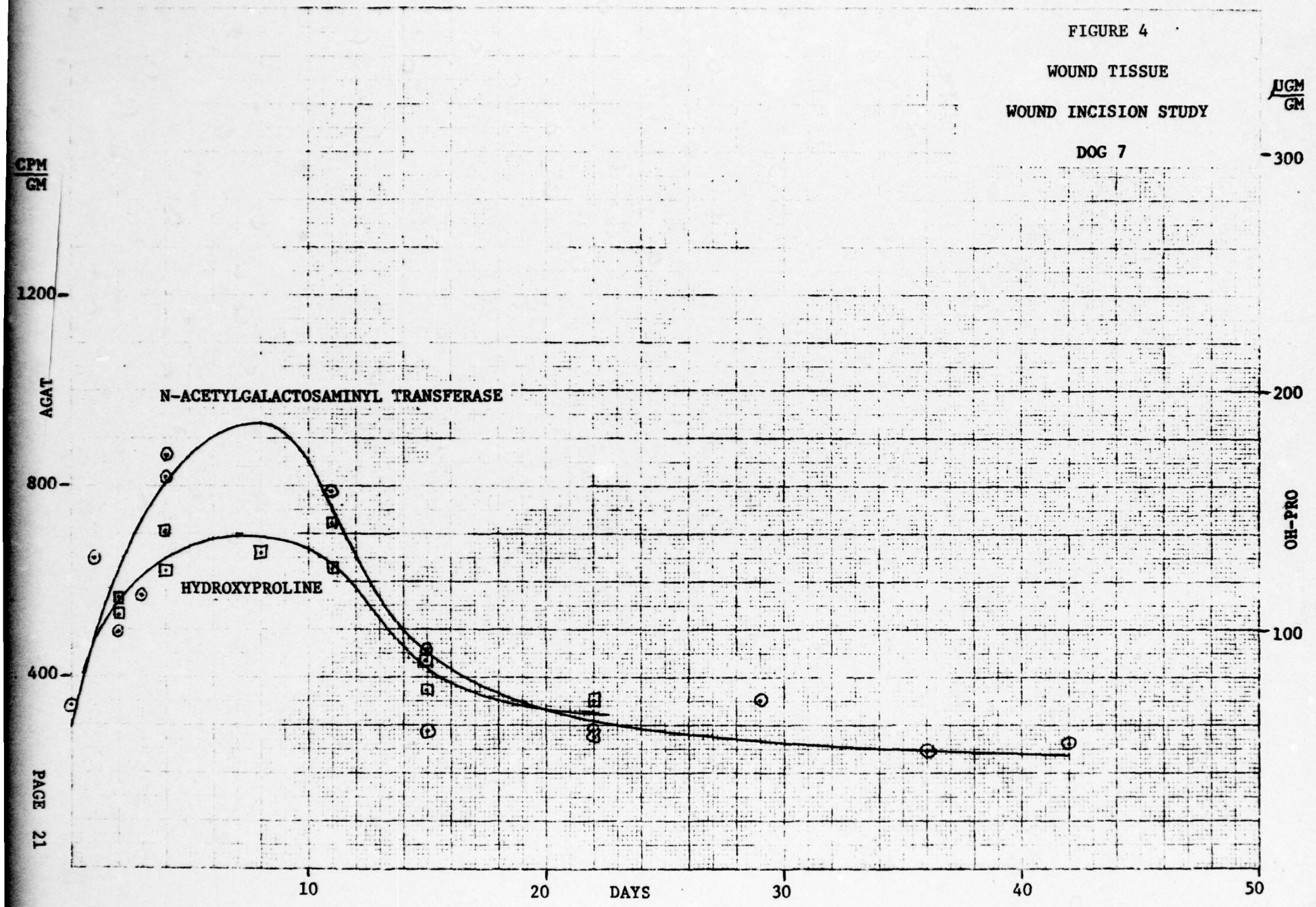
DOG 7

FIGURE 4

WOUND TISSUE

WOUND INCISION STUDY

DOG 7



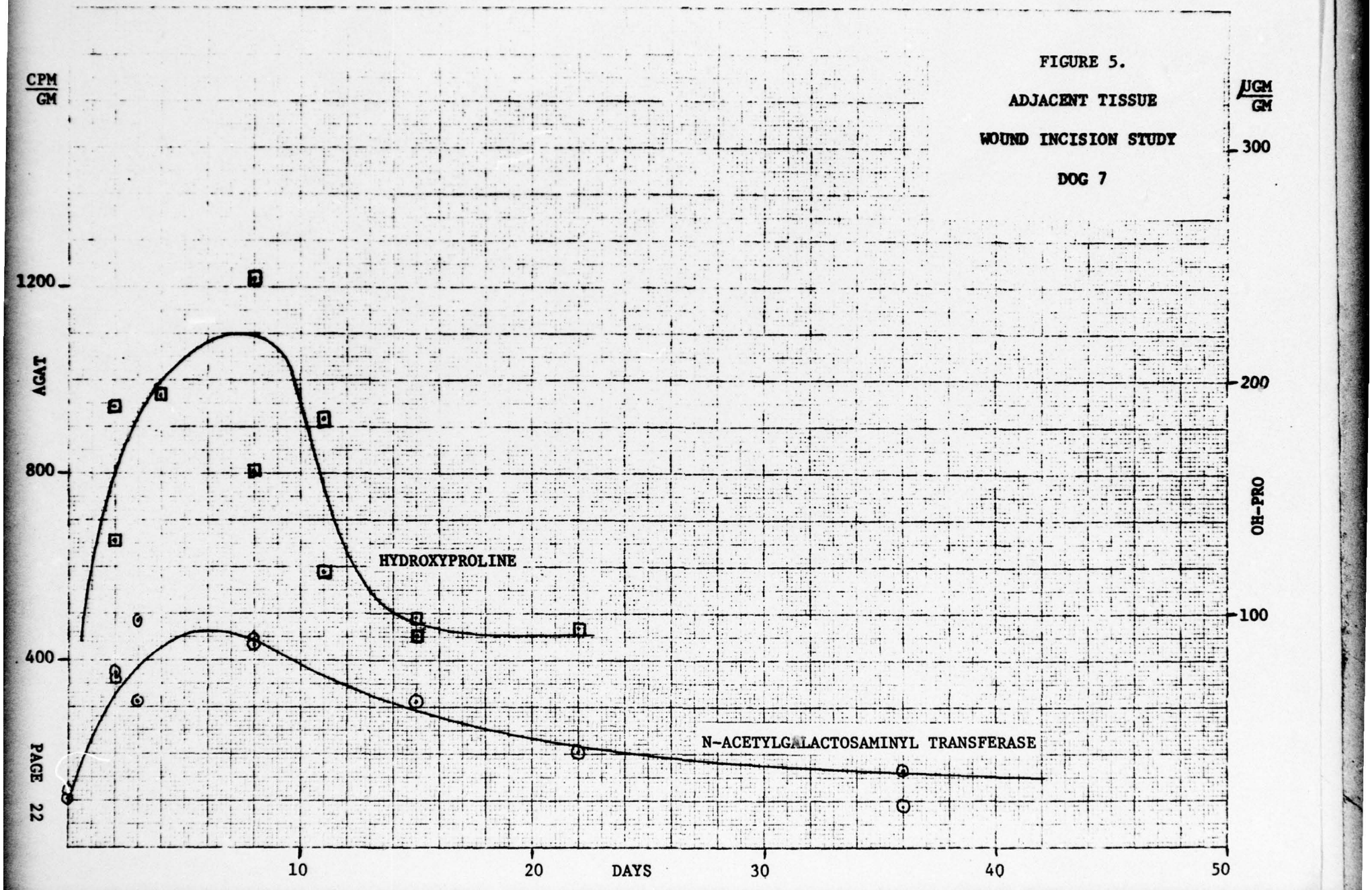


FIGURE 5.
ADJACENT TISSUE
WOUND INCISION STUDY
DOG 7
CPM/GM
AGAT
1200
800
400
μGM/GM
OH-PRO
300
200
100
HYDROXYPROLINE
N-ACETYLGALACTOSAMINYL TRANSFERASE
10
20
DAYS
30
40
50

FIGURE 6

N-ACETYLGALACTOSAMINYL TRANSFERASE

TURPENTINE TREATMENT

WOUND INCISION STUDY

DOG 10

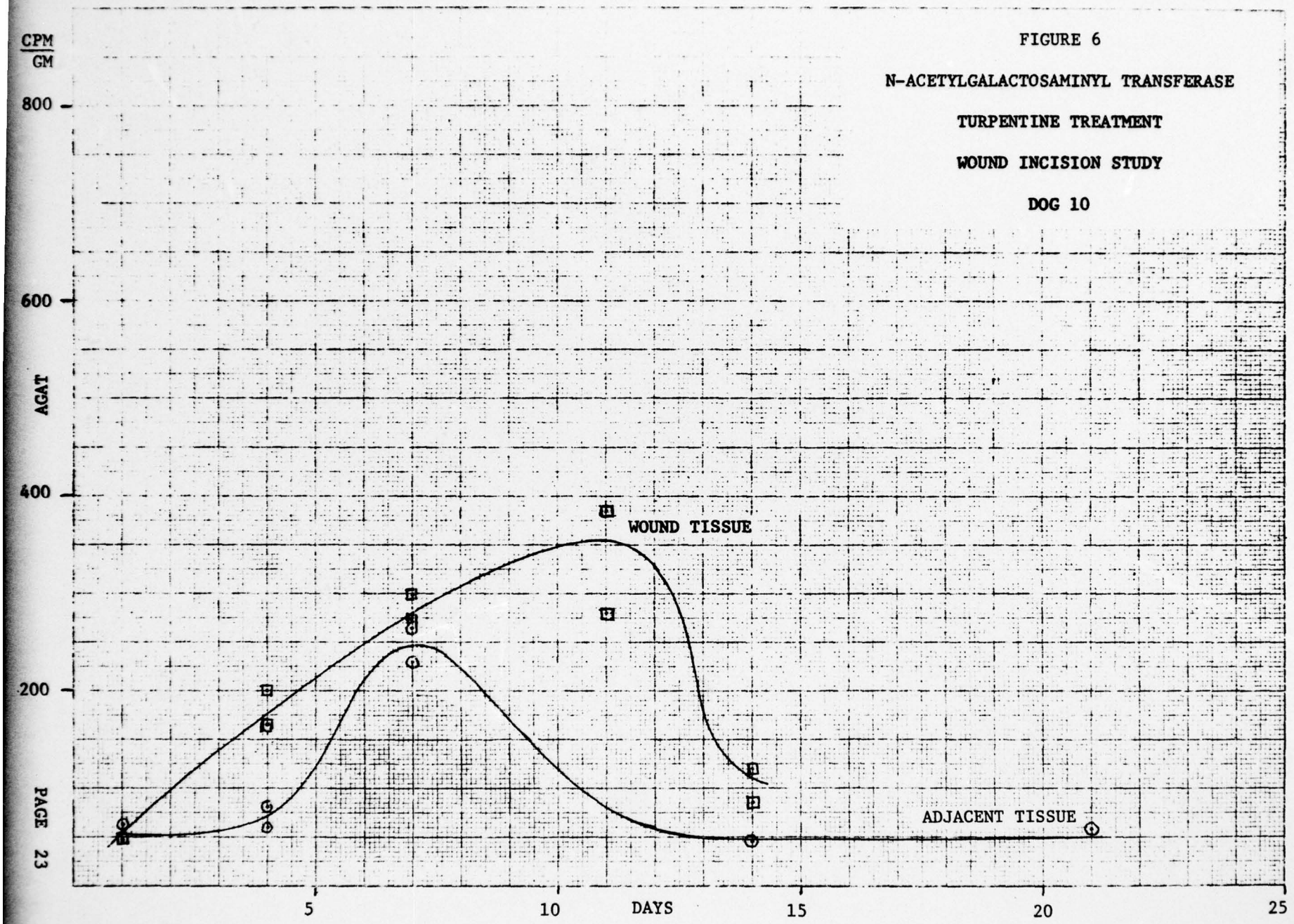


FIGURE 7

N-ACETYLGALACTOSAMINYL TRANSFERASE

WOUND TISSUE

WOUND INCISION STUDY

DOG 10

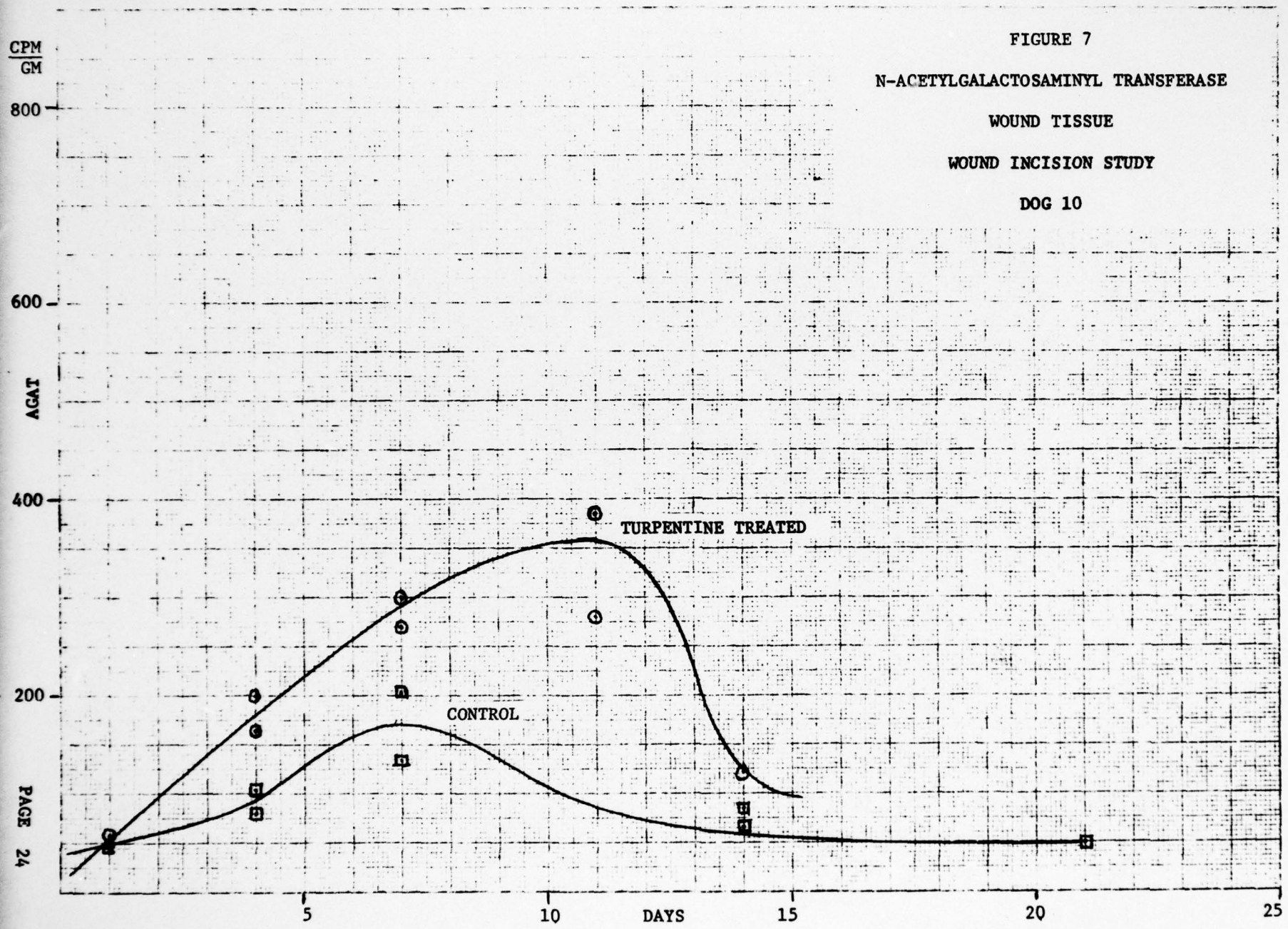


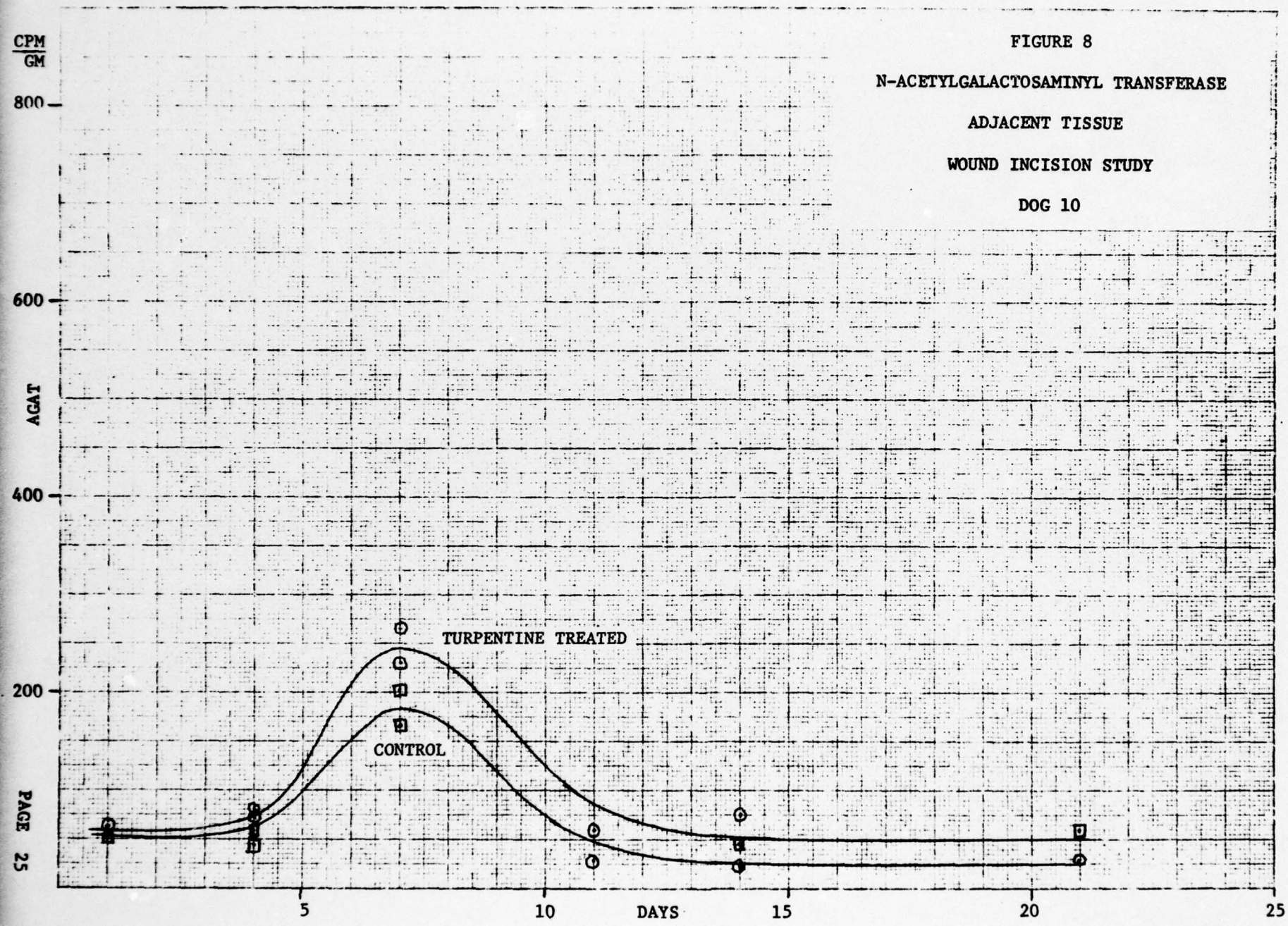
FIGURE 8

N-ACETYLGALACTOSAMINYL TRANSFERASE

ADJACENT TISSUE

WOUND INCISION STUDY

DOG 10



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